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Formation of Lipid-Bound Oligosaccharides Containing Mannose. Their Role in Glycoprotein Synthesis

(dolichol/glycolipid/GDP-Man/liver microsomes)

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ABSTRACT **Incubation of GDP-[^{14}C]mannose with liver microsomes and magnesium ions led to the formation of dolichol monophosphate mannose and of other acid-labile compounds that contain oligosaccharides. Formation of these compounds was enhanced by addition of an acceptor lipid in the same fractions of DEAE-cellulose chromatography where bound dolichol is found. Alkaline treatment of the oligosaccharides, obtained by acid methanolysis, followed by paper electrophoresis, gave rise to the formation of two positively charged substances believed to be formed by deacetylation of hexosamine residues. Incubation of the oligosaccharide-containing compounds with liver microsomes and manganese ions resulted in a transfer to endogenous protein. The role of dolichol derivatives in glycoprotein synthesis is discussed.**

Incubation of liver microsomes with UDP-Glc and dolichol-P leads to the formation of dolichol-P-Glc and a substance believed to be dolichol-PP-oligosaccharide (1, 2). This substance, which has been referred to as glucosylated endogenous acceptor, can also be obtained by direct transfer of glucose from dolichol-P-Glc and acts as donor in oligosaccharide transfer to protein (3).

The structure of the oligosaccharide portion of glucosylated endogenous acceptor is not known, but studies with the substance labeled in the glucose moiety have given some information. Measurements of molecular weight by ion exclusion gave values of 3500, and the rate of migration on paper chromatography corresponds to that of a maltooligosaccharide of about 17 units (4). The oligosaccharide, therefore, has around 20 monosaccharide units.

When the oligosaccharide prepared by acid methanolysis was subjected to electrophoresis, it behaved as an uncharged substance and on treatment with alkali, it gave rise to two positively charged substances (5). These became neutral after treatment with acetic anhydride under conditions that led to *N*-acetylation. From these facts it was tentatively concluded that the oligosaccharide of glucosylated endogenous acceptor contains two hexosamine residues.

In experiments where UDP-[^{14}C]GlcNAc was used as donor and dolichol-P as acceptor, it was found that substances that appear to be dolichol-PP-GlcNAc (6) and dolichol-PP-*N*,*N*'-diacetylchitobiose are formed (7). The transfer of both the sugar residue and phosphate from UDP-GlcNAc to a lipid had been described by Molnar *et al.* (8).

Abbreviation: R_G, ratio of migration of substance to migration of glucose.

These findings can be related to the fact that two *N*-acetylglucosamine residues are present in the inner portion of the oligosaccharide of many glycoproteins (9). In these, an *N*,*N*'-diacetylchitobiose residue is linked to the amide *N* of asparagine and chains of mannose residues are linked to it.

In experiments with GDP-Man as donor, lipid-bound mannose has been detected (10–12). Its formation was enhanced by addition of dolichol-P (6, 13), and the structure of the product has been unambiguously proved to be dolichol-P-Man by Hemming's group (14).

In previous experiments it was found that, besides dolichol-P-Man, other substances are formed which, when hydrolyzed with acid, yield products that migrate like oligosaccharides (4). These substances, which will be referred to as mannosylated endogenous acceptors, have now been studied in more detail.

MATERIALS AND METHODS

Dolichol-P was obtained as described (1) and purified up to the DEAE-cellulose step. 1 μl contained the dolichol-P extracted from 70 mg of pig liver (wet weight). Rat-liver microsomes were prepared as described by Moulé *et al.* (15).

Extraction of Mannosylated Endogenous Acceptors. Lipids formed by incubation with GDP-[^{14}C]Man were extracted as follows: 0.4 ml of methanol, 0.15 ml of 4 mM $MgCl_2$, and 0.6 ml of chloroform were added to the reaction mixture. After the sample was mixed and centrifuged, the lower phase and the interphase were washed with theoretical upper phase (chloroform–methanol–4 mM $MgCl_2$, 3:48:47, v/v). The protein interphase was extracted three times with coloroform–methanol–water (1:1:0.3, v/v) and the extract was mixed with the lower phase, dried on planchets, and counted.

Extraction of Acceptor Lipid. Pig liver (50 g) was ground with 100 ml of methanol and 150 ml of chloroform. The mixture was centrifuged, and the upper and lower phases were separated. The insoluble residue was extracted again with 50 ml of 4 mM $MgCl_2$, 100 ml of methanol, and 150 ml of chloroform and centrifuged. Both the lower phases and the insoluble residue were washed first with theoretical upper phase up to the point in which the solvent was free of color and then with $MgCl_2$-free theoretical upper phase. The lower phase was adjusted with methanol and water in order to give a chloroform–methanol–water mixture in the proportions 1:1:0.3 (v/v). The insoluble residue was extracted three times with

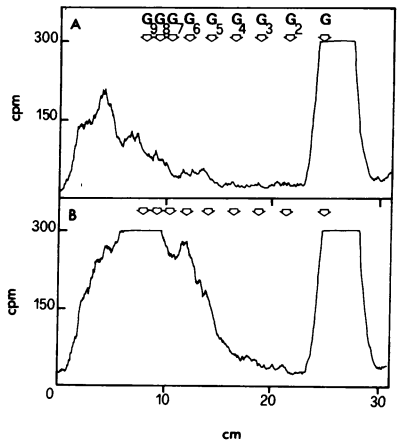

FIG. 1. (*A*) Formation of mannosylated endogenous acceptor from GDP-Man; (*B*) the same plus acceptor lipid. The incubation mixture contained 90,000 cpm of GDP-[^{14}C]Man (154 Ci/mol), liver microsomes (about 2 mg of protein), 0.01 M Mg-EDTA, 0.01 M $MgCl_2$, 0.1 M 2-mercaptoethanol, 0.1 M Tris-maleate (pH 7.7), and 0.5% sodium deoxycholate. Final volume, 50 μl. Added lipids were dried in the test tubes before the other components. Dolichol-P (20 μl) was added to both samples, and 0.3 ml of washed acceptor lipid was added to sample *B*. After 30 min at 30° the lipids were extracted, hydrolyzed with acid, and chromatographed on paper. *G*, glucose.

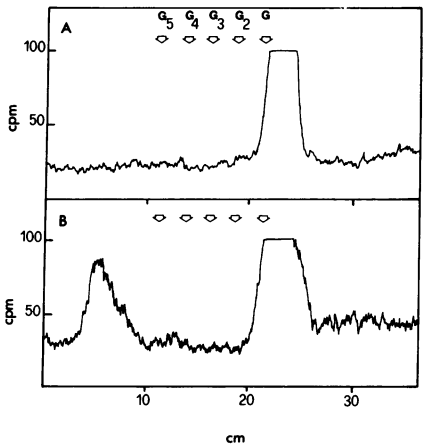

FIG. 2. Transfer of mannose from dolichol-P-Man. (*A*) Hydrolyzed dolichol-P-Man; (*B*) dolichol-P-Man incubated with acceptor lipid as follows: acceptor lipid (0.5 ml) and radioactive dolichol-P-Man (5000 cpm) were dried under reduced pressure with 0.5 μmol of Na-EDTA. Then buffer, mercaptoethanol, deoxycholate (1% instead of 0.5%), and enzyme (as in Fig. 1) were added. After incubation the lipids were extracted, hydrolyzed with acid, and chromatographed on paper. *G*, glucose.

chloroform–methanol–water (1:1:0.3, v/v). Both extracts were pooled, giving a final volume of 550 ml.

DEAE-Cellulose Chromatography. The lipid extract was poured into a 1.2 × 46-cm column of DEAE–cellulose (acetate form in chloroform–methanol–water, 1:1:0.3, v/v) and eluted with a linear gradient formed by mixing 150 ml of chloroform–methanol–water (1:1:0.3, v/v) and 150 ml of the same solvent containing 0.133 M ammonium formate. Under these conditions, dolichol-P-Glc emerged when about 148 ml had passed through the column and glucosylated endogenous acceptor, with about 193 ml. In some cases thymol blue was used as standard. It emerged with about 139 ml of solvent. When necessary, the fractions were washed free from ammonium formate by addition of chloroform and water so as to obtain a proportion of chloroform–methanol–water of 3:2:1. The upper phase was removed and the lower phase was washed repeatedly with the theoretical upper phase.

Mild Acid Hydrolysis. The labeled lipids were dried and suspended in water. HCl was added until thymol blue just turned red. The samples were then heated for 10 min at 100°.

Paper Chromatography and Electrophoresis. The solvent used for paper chromatography was 1-butanol–pyridine–water (4:3:4, v/v) (4). Paper electrophoresis was done in 5% formic acid or in 0.15 M carbonate–bicarbonate buffer (pH 9.2).

RESULTS

Formation of Mannosylated Endogenous Acceptor. When labeled GDP-Man was incubated with dolichol-P and liver microsomes, the main product formed was dolichol-P-Man. Thus, if at the end of the incubation, the lipid fraction was hydrolyzed with mild acid and the products were chromatographed on paper, most of the radioactivity migrated like mannose. A variable and usually small amount of slower moving radioactivity was also detectable in a position corresponding to oligosaccharides (Fig. 1*A*). Addition of acceptor lipids resulted in the appearance of a radioactive zone that migrated like the maltooligosaccharides of 5–16 units (Fig. 1*B*).

The substances running like oligosaccharides were eluted from the paper and submitted to acetolysis (2, 16). Paper chromatography of the products showed that besides mannose (R_G = 1.06), other compounds with the approximate mobility of di- and trisaccharide were formed (R_G = 0.9 and 0.77, respectively). In the same experiment, the R_G values for maltose and maltotriose were 0.88 and 0.79, respectively. The pattern was therefore as expected for the breakdown products of an oligosaccharide.

In order to find out if the donor for mannose transfer is GDP-Man directly or dolichol-P-Man, experiments were done in which the latter was incubated with the acceptor lipid. A considerable amount of radioactivity appeared in the oligosaccharides liberated by mild acid treatment (Fig. 2). Therefore, it seems that the transfer to at least some of the acceptors can take place directly from dolichol-P-Man.

DEAE–Cellulose Chromatography. It was reported that when radioactive dolichol-P-Glc and glucosylated endogenous acceptor were chromatographed on DEAE–cellulose they were neatly separated (2). This separation is believed to be due to the fact that the first compound is a monophosphodiester and the second a pyrophosphodiester.

When the lipids obtained by incubation of acceptor lipid, GDP-[^{14}C]Man, and liver microsomes were chromatographed on DEAE–cellulose, two peaks of radioactivity appeared in the position of dolichol-P-Glc and another in the region corresponding to glucosylated endogenous acceptor (Fig. 3). Mild acid hydrolysis of these fractions followed by paper chromatography showed that the first peak gave a product that migrated like mannose (Fig. 4*A*). The second peak was usually rather flat and in the experiment shown had two maxima. Elution and hydrolysis followed by paper chromatography showed that the radioactivity of peak B corresponded

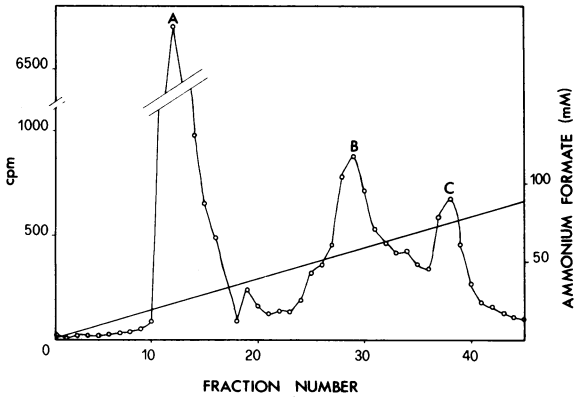

FIG. 3. DEAE–cellulose chromatography. A mixture of labeled mannosylated endogenous acceptor and dolichol-P-Man (about 350,000 cpm of total radioactivity) was separated by DEAE–cellulose chromatography. Fractions of 4.5 ml were collected. Aliquots of 0.3 ml were taken for measurement of radioactivity. Peaks *A*, *B*, and *C* were further treated (see Fig. 4).

to oligosaccharides that migrated like maltopentaose (Fig. 4*B*) and that of peak C to maltooligosaccharides of 9–16 glucose residues (Fig. 4*C*).

Alkaline hydrolysis of the product of acid methanolysis of glucosylated endogenous acceptor led to the appearance of positively charged substances. The change was interpreted as being due to the deacetylation of hexosamines (5). A similar experiment done with the acid methanolysis product of mannosylated endogenous acceptor showed that the product of methanolysis did not migrate during electrophoresis (Fig. 5). After 30 min of alkaline treatment, a substance that migrated to the negative pole appeared and after 60 min another substance that migrated at about twice the rate was detectable. The results were therefore the same as those obtained with glucosylated endogenous acceptor and are consistent with the idea that mannosylated endogenous acceptor contains two hexosamine residues.

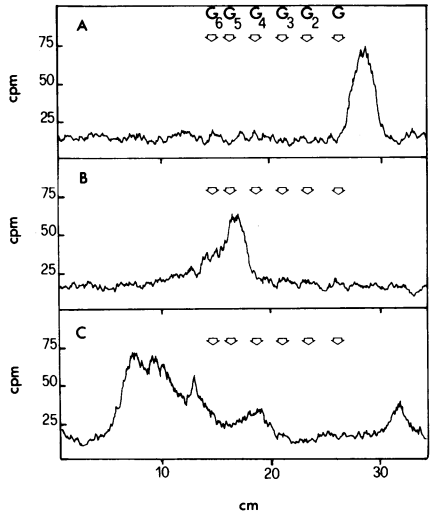

FIG. 4. Paper chromatography of the hydrolysis products of the substances separated by DEAE–cellulose chromatography. Aliquots of the labeled lipid fractions shown in Fig. 3 were hydrolyzed with mild acid and the sugars were separated by paper chromatography. Maltooligosaccharides were used as internal standards. *A*, *B*, and *C* correspond to the peaks indicated with the same letters in Fig. 3.

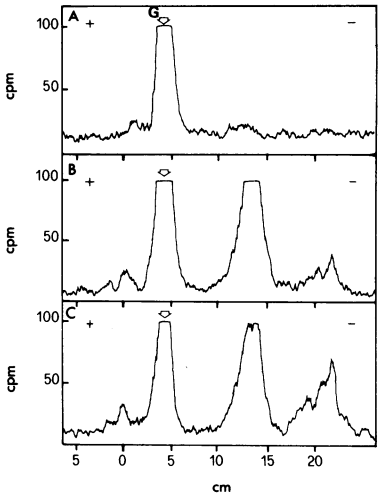

FIG. 5. Alkaline treatment of mannosylated endogenous acceptor oligosaccharides. Aliquots of a preparation of mannosylated endogenous acceptor purified by DEAE–cellulose chromatography (peak *B*, Fig. 3) were dried and treated with 0.1 M HCl in methanol for 60 min at 37°, then dried again and treated with 1 ml of 2 N KOH. Two 3000-cpm samples were heated at 100° for 30 min (*B*) and for 60 min (*C*). Sample *A* (1200 cpm) was not heated. The alkali was neutralized with perchloric acid, and the supernatant was subjected to electrophoresis in 5% formic acid (25 V/cm for 3 hr).

Transfer to Protein. The oligosaccharide-containing substances separated by DEAE–cellulose chromatography were tested for transfer of radioactivity to protein. A pool of fractions *B* and *C* of Fig. 3 were incubated with liver microsomes in the presence of manganese ions. The radioactivity in the lipid fraction decreased progressively while it increased in the protein fraction and somewhat increased in the water-soluble fraction (Fig. 6).

The denatured protein fraction was subjected to various treatments in order to confirm that the radioactivity is, in fact, covalently bound to a protein. Table 1 shows that after incubation with Pronase or crystalline trypsin, or treatment with alkali, the radioactivity became soluble in trichloroacetic acid. A mild acid treatment, with which mannosylated endogenous acceptor would break down completely, did not affect the product appreciably.

In another experiment the trichloroacetic acid-soluble product obtained by treatment with Pronase migrated to the positive pole when subjected to electrophoresis at pH 9.2 and to the negative pole in 5% formic acid. This is the behavior expected for a compound containing amino acids. The oligosaccharide of mannosylated endogenous acceptor was neutral under both conditions.

An experiment was done to determine if the protein obtained by transfer from mannosylated endogenous acceptor is soluble before denaturation. A reaction mixture prepared as described in the legend of Fig. 6 was incubated, diluted with 0.1 M Tris·HCl (pH 7.5) buffer and centrifuged 1 hr at 100,000 × *g*. The protein in the pellet and supernatant was fractionated and counted. Most of the radioactivity (85%) was found in the pellet. A similar result was obtained with protein prepared by transfer from glucosylated endogenous acceptor. In both cases, dilution with 1% Na-deoxycholate before centrifugation solubilized about 60% of the radioactive protein.

TABLE 1. *Action of enzymes, alkali, and acid on the insoluble radioactive product*

Treatment	Trichloroacetic acid	
	Insoluble	Soluble*
	cpm	
None	1,100	132
Pronase	185	665
Trypsin	263	760
Alkali	30	—
Acid	745	222

Alkaline degradation and Pronase digestion of the insoluble product were performed as described (3). Treatment with trypsin (3× crystallized) was done for 40 hr at 37° in 100 mM Tris-maleate (pH 7.8). Acid treatment consisted in adding enough HCl to the insoluble product so as to give pH 2 and then heating to 100° for 10 min. After the various treatments, microsomal protein was added as carrier followed by 10% trichloroacetic acid. The pellet was washed and counted as described in Fig. 6. The fraction soluble in trichloroacetic acid was extracted with ethyl ether and then counted in a gas-flow counter.

* Not corrected for self absorption.

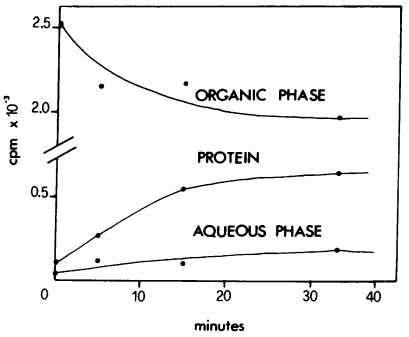

FIG. 6. Transfer to protein. A pool of mannosylated endogenous acceptor-containing fractions from the DEAE-cellulose column was freed from ammonium-formate. Aliquots (2.700 cpm) were dried and then suspended in 10 μl of 0.5 M Tris-maleate buffer (pH 7.7) and 5 μl of 5% Na-deoxycholate. Microsomal enzyme (25 μl containing about 3 mg of protein) was added and the reaction was then started by addition of 5 μl of 0.1 M $MnCl_2$. Incubation was done at 30°. The products formed were assayed as follows: 0.2 ml of 4 mM $MgCl_2$, 0.4 ml of methanol, and 0.6 ml of chloroform were added and the mixture was centrifuged. The upper aqueous phase was separated from the lower organic phase and from the denatured protein interphase. This extraction was repeated, and the denatured protein was then extracted with 1 ml of chloroform–methanol–water (1:1:0.3, v/v) which was added to the organic lower phases. After it was washed once with 0.5 ml of methanol, the protein residue was suspended in 0.2 ml of Protosol (New England Nuclear Corp.) and counted in a toluene-based scintillation mixture. The organic and aqueous phases were measured in a gas-flow counter.

DISCUSSION

The oligosaccharide-containing compounds formed by incubation of microsomes with GDP-Man are very similar to the compound formed with UDP-Glc. Both are acid labile and emerge in about the same position when chromatographed on DEAE-cellulose. The oligosaccharides of both give positively charged substances on alkaline treatment as if they had two acetyl-hexosamine residues. Paper chromatography showed that the mannose-labeled oligosaccharide is more polydisperse and of smaller size.

Formation of mannosylated endogenous acceptor is stimulated by an acceptor lipid which probably is a dolichol-pyrophosphate-oligosaccharide, since it was obtained from a fraction separated by DEAE-cellulose chromatography which was shown to contain bound dolichol-P (17) and in which bacterial prenol pyrophosphate derivatives are eluted (2).

Transfer of mannose to the acceptor lipid takes place either with GDP-Man or dolichol-P-Man as donors. One possibility is that dolichol-P-Man is a necessary intermediate in the transfer from GDP-Man; the other is that both compounds can act independently as donors.

A connection between mannosylated endogenous acceptor and the synthesis of glucosylated endogenous acceptor has not been experimentally established. However, with the results obtained now, it seems quite probable that a mechanism of the type detailed in the following reaction sequence is operative:

$$\text{UDP-GlcNAc} + \text{dolichol-P} \rightarrow \text{dolichol-PP-GlcNAc} + \text{UMP} \quad [1]$$

$$\text{UDP-GlcNAc} + \text{dolichol-PP-GlcNAc} \rightarrow \text{dolichol-PP-(GlcNAc)}_2 + \text{UDP} \quad [2]$$

$$\text{GDP-Man and/or dolichol-P-Man} + \text{dolichol-PP-(GlcNAc)}_2 \rightarrow \text{dolichol-PP-(GlcNAc)}_2(\text{Man})_x \text{ (mannosylated endogenous acceptor)} \quad [3]$$

$$\text{dolichol-P-Glc} + \text{dolichol-PP-(GlcNAc)}_2(\text{Man})_x \rightarrow \text{dolichol-PP-(GlcNAc)}_2(\text{Man})_x(\text{Glc})_2 \text{ (glucosylated endogenous acceptor)} \quad [4]$$

where $(\text{Man})_x$ stands for mannose oligosaccharides ranging from 3 to about 16 units. However, we cannot exclude that other sugar besides mannose are present.

The oligosaccharide moiety of mannosylated endogenous acceptor, like that of glucosylated endogenous acceptor, is transferred to a product that is insoluble in trichloroacetic acid but that becomes soluble after treatment with proteolytic enzymes. The product formed with the latter treatment behaved like an anphoteric substance when subjected to electrophoresis. Therefore, it is fairly certain that the oligosaccharide is transferred to protein.

Some considerations on the anomeric configuration of the compounds can be of interest. The sugar nucleotides in question are known to be α, and dolichol-P-Glc is probably β since it yields 1,6-anhydroglucosan on alkaline treatment (1). If all the reactions are assumed to occur with inversion, then the glucose residue in glucosylated endogenous acceptor should be α since dolichol-P-Glc is the donor. Dolichol-P-Man would be β and the mannose residues of the oligosaccharides would be α or β depending on whether the donor is dolichol-P-Man or GDP-Man, respectively. As to the innermost *N*-acetylglucosamine, it would be α when jointed to dolichol-PP and β after transfer to protein. The other *N*-acetylglucosamine would be transferred directly from UDP-GlcNAc and, therefore, becomes β as in *N,N'*-diacetylchitobiose.

The mannose oligosaccharides studied here seem to belong to the type found in glycoproteins bound to asparagine. On the basis of these facts, it may be assumed that the biosynthesis of this type of glycoproteins occurs by transfer of sugar residues to lipid so that the oligosaccharide is built up, joined to the lipid, and then transferred to the asparagine residue of a protein. It is possible that for formation of the finished glycoprotein, other glycosyl residues are added after the transfer from the lipid.

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